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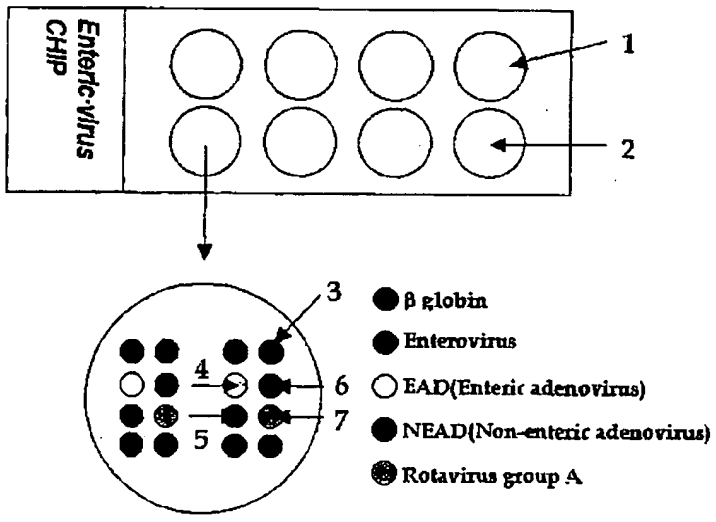
(54) Title: PROBE FOR DETECTION OF ENTERIC VIRUS DETECTION KIT AND METHOD FOR ENTERIC VIRUS WITH THE SAME



(57) Abstract: The present invention relates to a probe or probe set for detecting an enteric virus, a detection kit for an enteric virus comprising the probe or probe set, and a detection method for an enteric virus using the same. More particularly, the present invention provides an oligonulcleotide probe having a nucleic acid sequence as shown in SEQ ID NO. 1-15. In addition, the diagnosis kit for an enteric virus comprises a DNA chip with at least one probe that has nucleotides sequences complementary to DNA of an enteric virus, primers for amplifying a sample DNA by PCR, and means for labeling a sample DNA hybridized with the probe. When the invention can be applied to the detection of an infectious enteric virus in clinical samples, or to monitoring water contaminated with water-borne enteric viruses, a rapid and accurate result can be obtained.

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PROBE FOR DETECTION OF ENTERIC VIRUS, DETECTION KIT AND METHOD FOR ENTERIC VIRUS WITH THE SAME

BACKGROUND OF THE INVENTION

(a) Field of the invention

The present invention relates to a probe(s) for detecting enteric viruses, a detection kit comprising the probe(s), and a detection method for enteric viruses using the same. More specifically, the present invention provides probes for detecting enteric viruses including pan-enteroviruses, adenoviruses, rotaviruses, astroviruses, Norwalk viruses, Norwalk-like viruses, caliciviruses, and small round-structured viruses (SRSV), a detection kit comprising the probes, and a detection method for enteric viruses using the same.

(b) Description of the related art

The term "enteric virus" generally means a virus which primarily infects a human intestinal tract via mouth from the natural environment such as from water or soil, and then diffuses within a human body, thereby seriously influencing the individual involved. About 100 kinds of enteric viruses are known, most of them (about 67) being human enteroviruses (Glass RI, Bresee J, Jiang B, Gentsch J, Ando T, Fankhauser R, Noel J, Parashar U, Rosen B, Monroe SS. Gastroenteritis viruses: an overview. Novartis Found Symp 238:5-19; discussion 19-25, 2001; Morens, D. M., M. A.

Pallansch, and M. Moore. Polioviruses and other enteroviruses. *In* Belshe, (ed.), Text Book of Human Virology, Mosbey Year Books. 427-497, 1991).

It has been reported that there is a possibility of water-born enteric viruses contaminating water for home use, including drinking water, thereby causing disease. Recently, a great deal of money has been spent in supporting studies on monitoring and controlling of enteric viruses causing water-bone disease in drinking water sources and water for home use on a country-wide scale.

Viral enteritis is caused by a virus infecting intestines, and symptoms thereof include diarrhea, vomiting, headache, fever, and abdominal pain. The symptoms follow one or two days after infection and are maintained for 1-10 days. 3-5 billion cases and 5-10 million deaths are attributed to diarrhea each year (Walsh JA and Warren KS: Selective primary health care: An interim strategy for disease control in developing countries, N. Engl. J. Med. 302:967-974, 1979), the problem being more serious in developing countries.

Diarrhea is less serious than influenza and tuberculosis, but it affects daily life and industrial behavior, thereby causing large economic losses. Thus, about 1 billion dollars is spent per year in treating diarrheal patients in the United States (Ho M, et al.: Rotavirus as a cause of diarrheal morbidity and mortality in the United States, J. Infect. Dis. 158:1112-1116, 1988). At present, the viruses causing diarrhea are known to include rotaviruses, adenoviruses, caliciviruses, Norwalk viruses, Norwalk-like viruses, astroviruses, SRSV, reoviruses, etc. (F. Le Guyader, L. Haugarreau, L. Miossec, E. Dubois, and M. Pommepuy. Three-Year Study to Assess Human

Enteric Viruses in Shellfish. Appl. Envir. Microbiol. 66:3241-3248, 2000). In addition, the hepatitis A and E viruses, which are classified as enteric viruses, also cause liver disease. Viral diarrhea is usually transferred through physical contact with patients, and through food stuff contaminated by the virus. Contamination of food stuff can be caused by carefulness, for example, by a patient cooking without washing hands after using the restroom. Drinking water and shellfish can be infected by the virus contained in sewage including a patient's excreta.

Viral diarrhea occurs worldwide. Viruses often show epidermal characteristics, as with the rotavirus and astrovirus that are epidermal in winter. The adenovirus, however, is epidermal in all seasons. Nonbacterial collective diarrhea can be commonly caused by viruses occurring at collective feeding places such as schools, kindergartens, institutions for the aged, cruise ships, and dormitories.

Major diseases that the enteric viruses cause via primary infection and secondary infection, and the detecting and identifying method of the viruses, are summarized in the following Table 1.

[Table 1] Kinds of enteric viruses and disease

Virus	Classification	Genome type	Major disease	Diagnosing method
Norwalk virus	Caliciviridae	ssRNA	Acute gastroenteritis	Nucleic acid-based detection method
SRSV	caliciviridae	ssRNA	Acute gastroenteritis	Nucleic acid-based

				detection method
Enterovirus	Picornaviridae	ssRNA	Nonbacterial encephalomeningitis, encephalomyocarditis, encephalitis, epidermal hemorrhagic keratoconjunctivitis, foot-mouth disease, eruptive, flare	-Nucleic acid-based detection method - antibody neutralization test, - Fluorescent Antibody Detection
Rotavirus	Reoviridae	dsRNA	Diarrheal	Sequencing, antigen coagulation test
Astrovirus	Caliciviridae	ssRNA	Diarrheal	Sequencing
Adenovirus	Adeonoviridae	dsDNA	Diarrheal	Sequencing Fluorescent Antibody Detection
Hepatitis A virus	Picornaviridae	ssRNA	Acute hepatitis	-Nucleic acid-based detection method
Hepatitis E virus	Claiciviridae	ssRNA	Hepatitis	-Nucleic acid-based detection method

As shown in Table 1, the diseases caused by enteroviruses have a tendency to be epidermal worldwide, with symptoms of nonbacterial encephalomeningitis, encephalomyocarditis, encephalitis, epidermal hemorrhagic keratoconjunctivitis, and foot-mouth disease causing eruptive, flare, as well as intestine-related diseases. The methods of detection and identification for enteric viruses include i) a direct-detecting method of the enteric virus contained in feces and cerebrospinal fluid (CSF) of patients and concentrated water samples, ii) an antibody neutralization test after virus enrichment by inoculating the sample to a cultured cell, or iii) a detecting method consisting of the steps of amplifying the enriched virus through PCR or RT-PCR, and then hybridizing the PCR product with southern blot hybridization or sequencing.

The identification method using a specific antigen is accepted by the WHO as a standard method, but it has disadvantages in methodological and

economical aspects in that before an antigen-antibody reaction, the virus must be isolated and amplified, and in that the method can only be used for restricted field due to cross-reactivity. To resolve problems of an antibody neutralization test, the nucleic acid-based detection method has been widely used recently, but it has a problem in that when a clinical sample is directly used for detection, it is not possible to determine whether the virus is infectious or not.

Accordingly, when the infectiousness of the virus of interest is important for detecting the virus, the virus in a sample must be replicated or amplified by firstly infecting a cultured cell before being hybridized with a specific probe. Then, the viral genetic materials replicated in the cultured cell can be detected by hybridizing with a specific probe directly, or after amplification of the viral genetic materials through PCR or RT-PCR (LA Jaykus, R De Leon, and MD Sobsey: A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridization. Appl. Environ. Microbiol. 62:2074-2080. 1996). When the nucleic acid-based detection method is applied for detecting the enteric virus, the nucleotide sequence must be analyzed for subsequent identification, and then compared with that of a reference sequence.

Although the nucleic acid-based detection method is more or less advantageous in economic and experimental aspects compared with the antibody neutralization method, the method requires professional manpower, expensive experimental devices, and a large space. Thus, an identification and detection method for enteric viruses with high credibility and specificity

even for a large sample is still required.

SUMMARY OF THE INVENTION

The present invention provides probes for detecting enteric viruses which can be used for easy and rapid detecting of the enteric viruses without an antibody specific to enteric viruses or a sequencing analysis.

The present invention also provides a detection kit for enteric viruses comprising the probe(s) for an enteric virus.

The present invention also provides a preparation method of DNA chip for detecting an enteric virus.

The present invention also provides a DNA chip for detecting an enteric virus.

The present invention also provides a detection method of enteric virus with the probes for detecting an enteric virus.

In the first aspect of the present invention, the present invention provides a probe comprising a nucleotide sequence which can hybridize DNA of an enteric virus, which is selected from the group consisting of oligonucleotides having nucleotide sequences set forth in SEQ ID NO. 1 to SEQ ID NO. 15.

In the second aspect of the present invention, the present invention provides a process for preparing a DNA chip for detecting an enteric virus which comprises the steps of:

(a) preparing a 5' terminal amine-linked DNA probe which is at least one selected from the group consisting of nucleotide sequences set forth in

SEQ ID NO. 1 to SEQ ID NO. 15;

(b) affixing the DNA probe on an aldehyde-derivatized surface of a solid support; and

(c) reducing excessive aldehydes which are not reacted with the amine.

In the third aspect of the present invention, the present invention provides a DNA chip for detecting an enteric virus, comprising a probe or probe set selected from the group consisting of nucleotide sequences of SEQ ID NO. 1 to SEQ ID NO. 15.

In the fourth aspect of the present invention, the present invention provides a detection kit for an enteric virus, which comprises:

(a) a DNA chip comprising a probe or probe set selected from the group consisting of nucleotide sequences of SEQ ID NO. 1 to SEQ ID NO. 15;

(b) primers for amplifying DNA obtained from a clinical sample of an enteric virus; and

(c) means for labeling the amplified DNA hybridized with the probes of the said DNA chip.

In the fifth aspect of the present invention, the detection kit for the enteric virus is used for identifying, detecting, or genotyping an enteric virus that is selected from the group consisting of human pan-enterovirus, human enteric adenovirus, human rotavirus, astrovirus, calicivirus, Norwalk virus, Norwalk-like virus, and SRSV.

In the sixth aspect of the present invention, the present invention

provides a detection method for an enteric virus which comprises the steps of:

(i) amplifying DNA obtained from clinical samples with primers for amplifying the enteric virus;

(ii) hybridizing the amplified DNA with a DNA chip comprising a probe or probe set selected from the group consisting of nucleotide sequences of SEQ ID NO. 1 to SEQ ID NO. 15; and,

(iii) detecting the hybridization with the probe.

In the seventh aspect of the present invention, the present invention provides a detection method for an enteric virus which comprises the steps of:

(i) amplifying DNA obtained from clinical samples with primers for amplifying the enteric virus;

(ii) hybridizing the amplified DNA with a probe or probe set selected from the group consisting of nucleotide sequences of SEQ ID NO. 1 to SEQ ID NO. 15; and,

(iii) detecting the hybridization with the probe.

In the eighth aspect of the present invention, the present invention provides the detection method, wherein in step a), three kinds of viral nucleotide sequences comprising human pan-enterovirus, human enteric adenovirus, and human rotavirus are simultaneously amplified from the sample through triplex PCR or RT-PCR with primers derived from 5'-UTP of human pan-enterovirus, a hexon gene of human enteric adenovirus, and a nucleotide sequence encoding capsid glycoprotein VP7 of human rotavirus,

and then are hybridized with the probe or probe set.

In the ninth aspect of the present invention, the present invention provides the detection method, wherein in step a), seven kinds of viral nucleotide sequences comprising human pan-enterovirus, human enteric adenovirus, human rotavirus, astrovirus, calicivirus, Norwalk virus, Norwalk-like virus, and SRSV are simultaneously amplified in the sample multiplex PCR or RT-PCR with primers derived from 5'-UTP of human pan-enterovirus, a hexon gene of human enteric adenovirus, a nucleotide sequence encoding capsid glycoprotein VP7 of human rotavirus, a nucleotide sequence encoding a capsid protein of astrovirus, or nucleotide sequences encoding a capsid glycoprotein of calicivirus, Norwalk virus, Norwalk-like virus, and SRSV, and then are hybridized with the probe or probe set.

In the tenth aspect of the present invention, the present invention provides a primer for amplifying a nucleotide sequence of an enteric virus, which is selected from the group consisting of a nucleotide sequence of SEQ ID NO. 19, SEQ ID NO. 23, SEQ ID NO. 24, and SEQ ID NO. 29 to SEQ ID NO. 37.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a and 1b are examples of a detection kit for enteric viruses according to the present invention

Fig. 2 shows a result of detecting an enteric virus obtained from a water sample with the detection kit of the present invention.

Fig. 3a, 3b, and 3c show results of detecting a standard enterovirus with the detection kit of the present invention.

Fig. 4 shows a result of detecting a standard adenovirus with the detection kit of the present invention.

Fig. 5 shows a result of detecting a standard rotavirus with the detection kit of the present invention.

Figs. 6a, 6b, 6c, and 6d show results of detecting an enteric virus obtained from a clinical sample with the detection kit of the present invention.

Fig. 7 shows results of detecting standard enteric viruses including astrovirus, calicivirus, Norwalk virus, Norwalk-like virus, SRSV, and rotavirus with the detection kit of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Inventors of the present invention invented probes that can complementarily bind to nucleic acid (DNA or RNA) of seven (7) groups in total of human enteric viruses consisting of three (3) groups which can be cultured and four (4) groups which cannot be cultured, including enterovirus, adenovirus, rotavirus, calicivirus, Norwalk virus, Norwalk-like virus, SRSV, and astrovirus, and the use thereof.

The enteroviruses which constitute 67 kinds among 100 kinds of enteric viruses are also called pan-enteroviruses, and they are divided into 3 kinds of polioviruses, 23 kinds of Coxsackie virus A, 6 kinds of Coxsackie virus B, 31 kinds of echoviruses, and 4 other kinds of enteroviruses called enterovirus 68, 69, 70, and 71. Each enterovirus group shares a genotype-specific region which is located in a nucleic acid sequence at position 164-

526 bp of a highly conserved 5'-UTR(3, 16). The oligonucleotide probes (Entero 1, 2, and 3 as shown in Table 2) capable of detecting all the pan-enteroviruses are designed on the basis of the genetic characteristics conserved in subtypes, and can be used for detecting most of the pan-enteroviruses. The probes specific to the enteric viruses prepared according to the present invention are shown in Table 2 and SEQ ID NO. 1 to 3.

The adenoviruses include human enteric adenovirus type 31, human enteric adenovirus type 40, human enteric adenovirus type 41, and other kinds of adenoviruses. On the basis of a nucleotide sequence located in 18858-19158 bp of a hexon gene which can be used for classifying the serotypes of the adenoviruses (M Puig, et al., Appl. Environ. Microbiol. 60:2963-2970, 1994; N. Jothikumar, et al., J. Virol. Method. 55: 401-415, 1995), the probes for detecting adenoviruses can be designed to discriminate between enteric adenoviruses (Adeno 1 and Adeno 3 as shown in Table 2) and non-enteric adenoviruses (Adeno 2 as shown in Table 2). The probes specific to the adenoviruses prepared according to the present invention are shown in SEQ ID NO. 4-6.

In the rotavirus, serotype G contains capsid glycoprotein VP7, and serotype P contains VP4 which can be cleaved by proteinase. 14 kinds of rotavirus serotype G have been reported thus far, of which 10 kinds of serotype G can infect humans, and serotypes G1 to G4 are distributed widely (Estes MK et al., Microbiol. Rev. 53:410-449, 1989; Gentsch JR, et al. J. Infect. Dis.174:S30-S36, 1996). Based on the nucleotide sequence located in 1-392 bp of VP7, probes for detecting rotavirus are designed to detect the

serotype G (Rota 1, Rota 2, Rota 3, and Rota 4 as shown in Table 4). The probes specific to the rotaviruses prepared according to the present invention are shown in Table 2 and SEQ ID NO. 7-10.

The astroviruses cause 9-26% of viral diarrhea in developed and developing countries each year (Bon, F. P. et al., J. Clin. Microbiol. 37:3055–3058.1999; Svenungsson, B. A. et al., Clin. Infect. Dis. 30:770–778. 2000). Based on the nucleotide sequence located in 186-294-bp of a gene encoding a capsid protein, the probes for detecting 8 kinds of astroviruses are designed (Astro 1 as shown in Table 4).

In addition, calicivirus, Norwalk virus, Norwalk-like virus, and SRSV belonging to the caliciviridae classification have significant variations in genotype and phenotype (B.A. Lopman a,b, D.W. et al., J of Clin. Virol. 24:137–160. 2002; Shigeyuki Kojima a, et al., J of Virol Methods 100: 107–114. 2002). (질문: : **"Lopman a,b,"** 및 **"Shigeyuki Kojima a"**은 저자 이름인지요?) Based on the nucleotide sequence in -4 to 361-bp of the capsid-coding gene which is obtained by sequencing caliciviridae with VECTOR NTI (InforMax®), probes for detecting all kinds of caliciviridae are designed (Calici, Calici 1, Calici 2, and Calici 3 as shown in Table 4).

The underlined part in Table 2 represents a linker (spacer) sequence of a probe to increase the specificity of the probe.

[Table 2] Probe for detecting enteric viruses

VIRUS	Sequence (5' to 3')	Length	SEQ ID NO.
pan-enterovirus	Entero 1 : 5'amine-DSTACTTCGAGAARCCYAGTANCRCCWTG-3	29	1
	Entero 2 : 5'amine-CGGAACCGACTACTTTGGGTGTCCG-3'	25	2
	Entero 3 : 5'amine-GGCTGCGTTGGCGGCCTACCTATGGCT-3'	27	3
adenovirus	Adeno 1 : 5'amine-GCACGCSACCCACGATGTAACCACAGACAGCGTGC-3'	35	4
	Adeno 2 : 5'amine-GCACGCBACSCACGAYGTRACCACAGACMGCGTGC-3'	35	5
	Adeno 3 : 5'amine-CTGTGGCTCCGACCCACGATGTAACCACAG-3'	30	6
rotavirus	Rota 1 : 5'amine-TTAATGTATGGTATTGAATATACCACAATTCT-3'	32	7
	Rota 2 : 5'amine-ATATTATTGAATTATATATTAAAATCA-3'	27	8
	Rota 3 : 5'amine-ATGGACTACATAATTTATAGATTTTT-3'	26	9
	Rota 4 : 5'amine- CTAACggTTAgCTCCTTTTAATgTATggTA-3'	30	10
astrovirus	Astro 1 : 5'amine-GATCGTAAGAGGTCTAATCTTYCCCACCA-3	29	11
Norwalk virus Norwalk-like virus calicivirus SRSV	Calici : 5'amine-CAGYTGGTDCCRGAGGYWAATRCWK-3'	25	12
	Calici 1 : 5'amine-ATWGAYCCCTGGATWA-3'	16	13
	Calici 2 : 5'amine-AATTTTGTVCARGCMCC-3'	17	14
	Calici 3 : 5'amine-GGHGARTTYACNRTDTCCCC-3'	20	15

R(A,g): Y(C,T): M(A,C): K(g,T): S(g,C): W(A,T), V(A,C,g): H(A,T,C): B(g,T,C): D(g,A,T): N(A,g,C,T)

The probes, or a DNA chip comprising the probes, are useful for testing a clinical sample including enteric viruses, and monitoring water-borne enteric viruses in water.

In an embodiment of the present invention, the detection kit for enteric viruses comprises a DNA chip comprising the probe or probe set selected from the nucleotide sequences as set forth in SEQ ID NO. 1 to 15), primers for amplifying the enteric viruses, and means for labeling the amplified DNA to hybridize with the probes. Preferably, the probes used for the DNA chip can be a probe or probe set selected from the group consisting of nucleotide sequences of SEQ ID NO. 1 to 15.

The DNA chip can further comprise a probe for beta-globin as a position marker and for control of the hybridization reaction.

The primers for amplifying enteric viruses are derived from 5'-UTR of human pan-enterovirus, a hexon gene of human enteric adenovirus, a gene encoding capsid glycoprotein VP7 of human rotavirus, a gene encoding capsid protein of astrovirus, or a gene encoding capsid protein of calicivirus, Norwalk virus, or SRSV. Preferred primers are shown in Table 3.

The labeling step of the amplified DNA can be performed by amplifying the gene of enteric virus with the addition of Cy5 (Cyanine 5)-modified dNTP during PCR amplification, or by amplifying the gene with Cy5-modified primers. The biotin-binding materials can be used for the labeling means. The biotin-binding materials include streptavidine-R-phycoerythrin.

When Cy5 is used for labeling, the labeled product can be detected by analyzing the fluorescent signal with analyzing devices such as a confocal laser scanner without additional reaction, thereby providing efficient and sensitive results compared with biotin-binding materials. In addition, any labeling means which is known to an ordinarily skilled person in the field can be applied to the present invention, and they include, as examples, Cy3, Alexa 488, Alexa 532, EDANS(5-(2'-aminoethyl)amino-1-naphthalene sulfuric acid), tetramethylrhodamine (TMR), tetramethylrhodamine isocyanate (TMRITC), x-rhodamine, and Texas red.

The DNA chip for detecting enteric viruses can be prepared by linking an amine group to the 5' terminal of the DNA probes of the present invention (as shown in SEQ ID NO. 1 to 15), affixing the probes to an aldehyde-derivatized solid surface, and then reducing excessive aldehydes which are not reacted with the amine.

When Entero 1, Entero 2, or Entero 3 for pan-enteroviruses is applied to a DNA chip, it is preferable to spot them on a solid surface by mixing Entero 1 or Entero 3 with Entero 2. The Entero 1, 2, or 3 is designed from 238-266 bp, 532-556 bp, or 357-383 bp in a 164-599 bp position of 5'-UTR, respectively. The functionality of the probe is better for a short enterovirus gene. However, when Entero 2 is used in a mixture with Entero 1 or Entero 3, the functionality of the probe is better for a long gene of enterovirus. The probes for enteroviruses have superior functionality in a mixture. Entero 1 or Entero 3 is used in a mixture with Entero 2 at the ratio of 1:2-2:1, wherein the Entero 1 or Entero 3 is used alone or together. More preferably, Entero 1 or

Entero 3 can be used in a mixture with Entero 2 at the ratio of 1:1. When the ratio of Entero 1 or Entero 3 with Entero 2 in a mixture is out of this range, the probe has a low chance to hybridize with the target, thereby making detection difficult. In addition, Entero 1 can be mixed with Entero 3 at the ratio of 1:2-2:1, preferably 1:1.

Adeno 1 or Adeno 3 is separately aligned on a solid surface so as to not mix with Adeno 2. Adeno 1 and Adeno 3 are designed for enteric adenoviruses, and Adeno 2 is designed for non-enteric adenoviruses. Thus, the separate alignment of the probes can provide a method for differentiating between non-enteric adenoviruses and enteric adenoviruses.

Preferably, the probe for beta-globin can be affixed on the solid surface as a position marker and for control of the hybridization reaction. The affixing step of DNA probes to the aldehyde-derivatized solid surface can be performed via Schiff's base reaction between the amine and the aldehyde groups under the conditions of a temperature of 30 to 40°C and 70 to 100% humidity.

The aldehyde can be reduced by reducing agents such as $NaBH_4$.

Preferably, the concentration of probes which react with the aldehyde-derivatized solid surface ranges from 100 to 300 pmol/μl. When the concentration of probes is out of the range, the probe may not bind to the aldehyde.

The solid can be glass, silicon dioxide, plastics, or ceramics.

According to the present invention, it is possible to simultaneously detect various enteric viruses by simultaneously amplifying genes of various

enteric viruses with suitable primers, and applying the amplified genes to the probes of the present invention.

In an embodiment of the present invention, the amplified product can be obtained by simultaneously amplifying 7 kinds of the nucleotide sequences including human pan-enterovirus, human enteric adenovirus, human rotavirus, astrovirus, calicivirus, Norwalk virus, Norwalk-like virus, and SRSV in a sample with primers derived from 5'-UTP of human pan-enterovirus, a hexon gene of human enteric adenovirus, a gene encoding a capsid glycoprotein VP7 of human rotavirus, a gene encoding a capsid protein of astrovirus, or a gene encoding capsid glycoprotein of calicivirus, a Norwalk virus, Norwalk-like virus, and SRSV through multiplex PCR or RT-PCR, hybridizing with the probes, and then detecting the hybridization.

A gene of an enteric virus in a sample is amplified by addition of Cy5-modified dNTP through PCR to label the amplified gene. The gene labeled by Cy5 can be detected by analyzing the fluorescent signal with a confocal laser scanner.

The present invention is further shown in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Preparation of PCR primers

Parts of genomes of enterovirus, adenovirus, rotavirus, calicivirus, Norwalk virus, SRSV, and astrovirus were amplified with the primers as shown in Table 3 and SEQ ID NO. 16 to 37.

[Table 3] PCR Primer used in Example

Primer	Position	Sequence	Length	SEQ ID NO	비고
EV 1	164-184	5'-CAAGCACTTCTGTTTCCCCGG-3'	21	16	Highly-conserved 5'-UTR
EV 2	599-578	5'-ATTGTCACCATAAGCAGCCA-3'	20	17	
EV 3	526-511	5'-CTTGCGCGTTACGAC-3'	15	18	
NEO	436-473	5'-GAATGCGGCTAATCCCAAC-3'	19	19	
RV 1	1-28	5'-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3'	28	20	capsid glycoprotein VP 7 fragment
RV 2	376-392	5'-GATCCTGTTGGCCATCC-3'	17	21	
RV 3	51-71	5'-GTATGGTATTGAATATACCAC-3'	21	22	
RV 4	215-240	5'-GTCCATTGATCCTGTTATTGGTAAAT-3'	26	23	
RV 5	292-314	5'-GCTTCWGYAGGGTARTAYARRCA-3'	23	24	
AD 1	18858-18883	5'-GCCGCAGTGGTCTTACATGCACATC-3'	25	25	ORF, hexon gene
AD 2	19136-19158	5'-CAGCACGCCGCGGATGTCAAAGT-3'	23	26	
AD 3	18937-18960	5'-GCCACCGAGACGTACTTCAGCCTG-3'	24	27	
AD 4	19051-19079	5'-TTGTACGAGTACGCGGTATCCTCGCGGTC-3'	29	28	
AS 1	186-204	5'-CTTCCCAARTTTGTTAGG-3'	18	29	capsid protein
AS 2	275-294	5'-CAGTAATCACAACAACTCRT-3'	20	30	
NV 1	1-22	5'-ATGAWGATGGCGTCKAAKGACG-3'	22	31	capsid protein
NV2	295-316	5'-CACYTGCATAMCCATTRTACAT-3'	22	32	
NV 3	295-316	5'-TGCCMACCCAGCCATTATACAT-3'	22	33	
NV 4	342-361	5'-CCRGCNGTRAAVGCKTTNCC-3'	20	34	
NV 5	-4-18	5'-GTAAATGATGATGGCGTCTAAG-3'	22	35	
NV 6	115-176	5'-TTATTSATWATCCAGDGRTCAAT-3'	22	36	
NV 7	184-203	5'-GGGGAHAKNGTKAAKTCDCC-3'	20	37	

In addition to a PCR of pan-enterovirus, human enteric adenovirus type 31, human enteric adenovirus type 40, human enteric adenovirus type 41, and human rotavirus were simultaneously amplified through triplex RT-PCR, and then hybridized with a DNA chip of the present invention to simultaneously detect 3 kinds of enteric viruses.

Example 2: Amplification of enteric viral genes from samples

2-1: Separating the enteric viral genes from sample

The enteric viral genes were separated from a clinical sample and a water sample as follows:

<Clinical sample>

Clinical materials and cerebrospinal fluid of patients who were considered to be infected by an enteric virus were donated from the laboratory of the enteric virus division of virology at the Korean National Institute of Health to perform the following experiments.

The clinical materials were preserved at 4℃. The clinical materials were suspended in PBS (pH 7.4) at 10 v/v%. 300 ㎕ of suspended clinical materials had 320 ㎕ of 6M guanidium isothiocyanate (GITC) and 10 ㎕ of RNaid silica matrix added thereto.

To separate the nucleic acid of the virus, the silica matrix was placed at room temperature for 10 minutes. The precipitated materials were washed with a washing buffer solution (Bio 101 inc. washing solution of RNaid kit) 3 times, and then dried completely under vacuum. The dried nucleic acids were

solubilized in 25 ㎕ of distilled water for subsequent RT-PCR or PCR.

100 ㎕ of a CSF sample was solubilized in 400 ㎕ of blood lysate (GITC, glycogen, and dithiothreitol in a Tris buffer solution) and then placed at room temperature for 10 minutes. The resultant sample was precipitated with the addition of 500 ㎕ of isopropanol and then centrifuged at 16,000×g, at 4℃ for 10 minutes, to precipitate only the nucleic acid. The precipitate was washed with 750 ㎕ of 70% ethanol, dried, and suspended in a bicine buffer solution containing manganese acetate and potassium acetate to use for RT-PCR.

<Water sample>

Water samples including untreated water, purified water, and domestic water were obtained from the laboratory of molecular virology of the Department of Biology in Kyunghee University in Seoul, Korea. To perform pre-culture PCR, the water samples were dripped off and concentrated. 200 ㎕ of the concentrated samples were reacted with proteinase K (0.5 ㎍/㎕, GIBCO BRL) and sodium dodecyl sulfate (1%, SIGMA) at 37℃ for 30 minutes, and then 100 ㎕ of a 1×TE buffer were added thereto. The resultant solutions were well mixed with samples that were reacted with phenol-chloroform-isopropanol (PCI, 25:24:1, SIGMA) in the same volume, and then centrifuged at 7000×g for 10 minutes.

The supernatant solution was transferred to a new tube, and the above reactions were repeated after the addition of a 1× TE buffer. The separated supernatant solution was eluted with chloroform, incubated at

room temperature for 1 hour with the addition of isopropanol at the same volume to prevent the sodium phosphate from precipitating, and then centrifuged at 7000×g for 30 minutes. The resultant precipitates were dried completely, and solubilized in 10 ㎕ of sterile triple-distilled water for subsequent RT-PCR.

To perform Integrated Cell Culture (ICC-PCR), the concentrated water samples were inoculated on a BGM cell line (Africa green monkey kidney cell, EPA in U.S.A.) according to the total culturable virus assay (TCVA) suggested by the U.S. EPA to amplify the virus in the cell. Then, the supernatant was removed from the culture, and the culture cell was washed with 4×PBS 3 times, cultured at room temperature for 15 minutes with the addition of an RNA extraction solution (50 mM sodium acetate, pH 5.2; 0.6%SDS; 1mM EDTA; proteinase K 60 ㎍/ml), and then cell lysis was performed. The cell lysate was incubated at 56℃ for 5-10 minutes, and centrifuged at 12,000×g, at 4℃ for 10 minutes, with the addition of phenol at the same volume to obtain a supernatant. The supernatant was mixed with a PCI solution at the same volume, and then the RNA contained in the cells was separated according to the same method as described above. The separated RNA was precipitated and purified with the addition of 100% ethanol at 5 times its volume. The RNA was again separated by treating the suspended RNA with the PCI solution, and then precipitated and purified with the addition of ethanol. The purified RNA was used as a PCR template. ICC-PCR with the cell lysate was performed under the same conditions as with the pre-culture PCR.

2-2: Amplifying sample DNA

In this Example, PCR or RT-PCR was performed as follows:

cDNA synthesis was performed by using a 1× random primer (Gibco BRL) according to the Gibco BRL's Superscript II reverse transcriptase manual. The reaction conditions were that cDNA complementary to viral RNA was synthesized at 25℃ for 20 minutes, and then at 42℃ for 1 hour, it was heated at 95℃ for 5 minutes to inactivate the reverse transcriptase, and then the reaction was performed under the following condition. The multiplex PCR in this Example was intended to amplify the nucleotide sequences of enterovirus, rotavirus, and adenovirus.

To obtain Cy5-labeled amplified DNA samples, the synthesized cDNA was amplified and labeled by using primers as shown in Table 2 with addition of Cy5 (dUTP or dCTP).

In the first step of amplification, the PCR was performed with 100 ㎕ or 50 ㎕ of 1 mM $MgCl_2$ with sense primer 1 (10 p㏖) and anti-sense primer 2 (10 p㏖). In the second step of the PCR (semi-nested PCR or nested PCR), 1/100 of the sample was amplified with sense primer 1 and anti-sense primer 3, or with sense primer 2 and anti-sense primer 3.

The PCR was performed by using a hot starter with 3 early cycles of denaturation for 30 seconds at 94℃, primer annealing for 30 seconds at 50℃, and extension for 30 seconds at 72℃. This was followed by 27 cycles of denaturation for 15 seconds at 94℃, primer annealing for 15 seconds at 50℃,

and extension for 20 seconds at 72℃, and then further extension for 2 minutes at 72℃. The amplification condition of the second step was performed under the same conditions as the first step.

The samples obtained from the water sample and the clinical sample were amplified with primers as shown in Table 3, and the amplified products of the enteric viral samples are shown in Table 4.

[Table 4]

Virus	PCR product (used primer set)	
Enterovirus	435bp(EV 1, 2)	362bp(EV 1, 3) 163bp(EV 2/NEO)
Rotavirus	392bp(Rota 1, 2) 342bp(Rota 2, 3)	314bp(Rota 1, 5) 189bp(Rota 2, 4)
Adenovirus	300bp(AD 1, 2)	142bp(AD 3, 4)
Astrovirus	109 bp(AS 1, 2)	-
Norwalk virus, Norwalk-like virus Calicivirus SRSV	361(NV 1, 4)	316(NV 1, 2,3) 179(NV5, 6)

Example 3: Preparation of DNA chip

To detect the genetic fragments of enteric viruses prepared from Example 2, a detection kit comprising the probes (shown in Table 2) was prepared. Each probe for detecting enteric viruses, such as mixed probes (6) of Entero 1 and 3 for detecting enterovirus were fixed, and Adeno 1 (4) and Adeno 2 (5) to differentiate the adenoviruses were fixed respectively onto an

aldehyde-derivatized silylated slide (1) consisting of eight reaction chambers (2). Rota 1, 2, 3, or 4 for detecting rotavirus (7), and astro 1 for detecting astrovirus (9) were spotted. Calici, Calici 1, Calici 2, or Calici 3, or mixed probes thereof at the ratio of 1:198 for detecting calicivirus, Norwalk virus, Norwalk-like virus, and SRSV were spotted. The probe of beta-globin (3) as a position marker and control of cross-reactivity was fixed as shown in Figs. 1a and 1b.

Each probe as prepared above was used at a concentration of 200 pmol/$\mu\ell$, and spotted onto an aldehyde-derivatized silylated slide, followed by performing Schiff's base reaction under the conditions of a temperature of 30-40℃ and 70-100% of humidity. Then, the slide was treated with an $NaBH_4$ solution (0.1g $NaBH_4$, 30ml phosphate buffered saline (PBS), 10ml ethanol) for 5 minutes to reduce excessive aldehydes not reacted with amine, followed by washing with triple-distilled water and drying.

Example 4: Detecting the samples

This Example was performed by analyzing the amplified samples of Example 2 with the DNA chip of Example 3 to show the usefulness of the probes of the present invention.

The hybridization and detection reactions were performed by the following methods.

The hybridization reactions were performed with small amounts of a PCR product. 20 $\mu\ell$ of a triplex PCR product and 5 $\mu\ell$ of a beta-globin PCR

product were diluted in 15 μℓ of distilled water, incubated with the addition of 4 μℓ (1/10 vol.) of a denaturation solution (3 N NaOH) at room temperature for 5 minutes, and incubated on ice for 5 minutes with the addition of 2 μℓ (1/20 vol.) of 1 M Tris-HCl (pH 7.2) and 4 μℓ (1/10 vol.) of a renaturation solution (3 N HCl) to neutralize the denaturation solution. The neutralized solutions had 50 μℓ of 12X SSPE (in case of 100 μℓ of cover slip) added thereto, as well as 0.5 μℓ of 10% SDS, and they were poured into holes of slides covered with cover slips (4-well chamber) while preventing air bubbles from occurring. The samples were incubated in a wet incubator at 40℃ for 2 hours to perform a cross-reaction. In the absence of a wet incubator, the humidity could be maintained by adding water to the incubator.

To detect hybridization, the cover slips were removed after termination of the cross-reaction. The slips were washed by agitating in 3X SSPE for 2 minutes and 1X SSPE for 2 minutes at about 50 rpm, and air-dried. Color signals were detected with a confocal laser scanner (GSI Lumonics. Inc.), and the results are shown in Fig. 2 to Fig. 7.

As shown in Fig. 2 to Fig. 7, only the probe mixture of Entero 1 and Entero 2 in the ratio of 1:1 (6) showed strong signals to poliovirus 1, 2, and 3 (Fig. 3a), Coxsackie virus B2, 3, 4, 5 (Fig. 3b), echovirus 7, 11, 24, and 30 (Fig. 3c). As shown in Fig. 4, Adeno 1 (4) and Adeno 2 (5) showed strong signals to adenovirus types 41 and 5, respectively. As shown in Fig. 5, Rota probe (7) showed strong signals to human rotavirus. As shown in Fig. 2 and Fig. 6, Adeno 1 (4) showed strong signal to the clinical sample, and the

mixture probe (6) of Entero 1 and Entero 2 in the ratio of 1:1 showed a strong signal to the water sample (JCW).

By sequencing the sample where the human enteric adenovirus was detected, nucleic acid sequences of adenovirus types 41 and 31 were confirmed. The result is shown in Table 5.

As shown in Fig. 7(A), Astro 1 (9) showed a signal to human astrovirus. Only mixed probes (8) of Calici, or Calici 1 and Calici, or Calici 2 and Calici, or Calici 2 and Calici 3 in the ratio of 1:1 showed strong signals to calicivirus, Norwalk virus, Norwalk-like virus, and SRSV.

[Table 5]

The sequence analysis and DNA chip analysis of clinical samples

sample	Sequence analysis	DNA chip analysis of the present invention
1647	adenovirus type 41	Enteric adenovirus
1691	adenovirus type 31	Enteric adenovirus

As a reference, the above experiments were repeated on poliovirus types 1, 2, and 3; Coxsackie viruses B2, 3, 4, and 5; and echoviruses 7, 11, 24, 30, which were classified into pan-enterovirus. Adenovirus types 5 and 41, classified into adenovirus, rotavirus, astrovirus, and viruses classified to caliciviridae were obtained from the Laboratory of Molecular Virology of Kyunghee University, Seoul, Korea and the Laboratory of Enteric Viruses in the Department of Virology in the National Institute of Health (Seoul, Korea). The results are shown in Fig. 3 to Fig. 7.

According to the Examples, probes for detecting the enteric viruses could be applied to a method of detecting various kinds of enteric viruses with accuracy and rapidity. In particular, adenoviruses including human enteric adenovirus were only detected by sequencing the nucleic acid prior to the present invention. However, the adenovirus can be detected easily and directly according to the present invention without sequencing.

In the present invention, the probes for detecting enteric viruses, and the detection kit comprising the probes, can provide a method of monitoring water-borne enteric viruses in water and clinical samples accurately and rapidly.

What is claimed is:

1. A probe comprising a nucleotide sequence which can hybridize DNA of an enteric virus, which is selected from the group consisting of oligonucleotides having nucleotide sequences set forth in SEQ ID NO. 1 to SEQ ID NO. 15.

2. A process for preparing a DNA chip for detecting an enteric virus which comprises the steps of:

(a) preparing a 5' terminal amine-linked DNA probe which is at least one selected from the group consisting of nucleotide sequences set forth in SEQ ID NO. 1 to SEQ ID NO. 15;

(b) affixing the DNA probe on an aldehyde-derivatized surface of a solid support; and

(c) reducing excessive aldehydes which are not reacted with the amine.

3. The process for preparing a DNA chip according to claim 2, wherein the probe of SEQ ID NO. 2 and at least a probe selected from the group consisting of the probe of SEQ ID NO. 1 and the probe of SEQ ID NO. 3 are mixed at the ratio of 1:2-2:1.

4. The process for preparing a DNA chip according to claim 2, wherein the probe of SEQ ID NO. 4 is aligned such that it does not mix with the probe of SEQ ID NO. 5 or the probe of SEQ ID NO. 6.

5. The process for preparing a DNA chip according to claim 2, wherein the affixing step of a DNA probe to an aldehyde-derivatized solid surface in step (b) is performed via Schiff's base reaction between the amine and the aldehyde group under the condition of a temperature of 30 to 40°C and 70 to 100% humidity.

6. The process for preparing a DNA chip according to claim 2, wherein the reducing step of aldehyde is performed with $NaBH_4$.

7. The process for preparing a DNA chip according to claim 2, wherein a concentration of probes that react with the aldehyde-derivatized solid surface ranges from 100 to 300 pmol/μl.

8. A DNA chip for detecting an enteric virus, which comprises a probe or probe set having a nucleotide sequence that can hybridize DNA of the enteric virus, the probe or probe set being selected from the group consisting of oligonucleotides having nucleotide sequences set forth in SEQ ID NO. 1 to SEQ ID NO. 15.

9. The DNA chip according to claim 8, wherein the DNA chip further comprises a probe for beta-globin.

10. The DNA chip according to claim 8, wherein the DNA chip is

prepared by the process as defined in any one of claims 2-7.

11. A detection kit for an enteric virus, which comprises:

(a) a DNA chip comprising a probe or probe set selected from the group consisting of nucleotide sequences set forth in SEQ ID NO. 1 to SEQ ID NO. 15;

(b) primers for amplifying DNA obtained from a sample of an enteric virus; and

(c) means for labeling the amplified DNA hybridized with the probes in the DNA chip.

12. The detection kit according to claim 11, wherein the enteric virus is at least one selected from the group consisting of human pan-enterovirus, human enteric adenovirus, human rotavirus, astrovirus, calicivirus, Norwalk virus, Norwalk-like virus, and small round-structured viruses (SRSV).

13. The detection kit according to claim 11, wherein the DNA chip is as defined in claim 8 or 9.

14. The detection kit according to claim 11, wherein the DNA chip is as defined in claim 10.

15. The detection kit according to claim 11, wherein the primer is selected from the group consisting of nucleotide sequences of SEQ ID NO. 16 to SEQ ID NO. 37.

16. The detection kit according to claim 11, wherein the means for labeling is selected from the group consisting of Cy5, Cy3, EDANS (5-(2'-aminoethyl)amino-1-naphthalen sulfuric acid), tetramethylrhodamine (TMR), tetramethylrhodamine isocyanate (TMRITC), a biotin-binding material, and Texas red.

17. The detection kit according to claim 16, wherein a sample DNA is labeled by amplifying it with the addition of a Cy5-modified dNTP through PCR, or by amplifying it with a Cy5-modified primer.

18. The detection kit according to claim 16, wherein the biotin-binding material is streptavidine-R-phycoerythrin.

19. A detection method for an enteric virus which comprises the steps of:

(i) amplifying DNA obtained from clinical samples with primers for amplifying the enteric virus;

(ii) hybridizing the amplified DNA with a DNA chip comprising a probe or probe set selected from the group consisting of nucleotide sequences of SEQ ID NO. 1 to SEQ ID NO. 15; and,

(iii) detecting the hybridization with the probe.

20. The detection method according to claim 19, wherein the primer is selected from the group consisting of nucleotide sequences of SEQ ID NO. 16 to SEQ ID NO. 37.

21. The detection method according to claim 19, wherein the DNA chip is the DNA chip as defined in claim 8 or 9.

22. The detection method according to claim 19, wherein the DNA chip is the DNA chip as defined in claim 10.

23. The detection method according to claim 19, wherein in step a), three kinds of viral nucleotide sequences comprising human pan-enterovirus, human enteric adenovirus, and human rotavirus are simultaneously amplified from the sample through triplex PCR or RT-PCR with primers derived from 5'-UTP of human pan-enterovirus, a hexon gene of human enteric adenovirus, and a nucleotide sequence encoding a capsid glycoprotein VP7 of human rotavirus, and then are hybridized with the probe or probe set.

24. The detection method according to claim 19, wherein in step a), seven kinds of viral nucleotide sequences comprising human pan-enterovirus, human enteric adenovirus, human rotavirus, astrovirus, calicivirus, Norwalk virus, Norwalk-like virus, and SRSV are simultaneously amplified in the sample multiplex PCR or RT-PCR with primers derived from 5'-UTP of human pan-enterovirus, a hexon gene of human enteric adenovirus, a nucleotide sequence encoding a capsid glycoprotein VP7 of human rotavirus, a nucleotide sequence encoding a capsid protein of astrovirus, or nucleotide sequences encoding a capsid glycoprotein of calicivirus, Norwalk virus,

Norwalk-like virus, and SRSV, and then are hybridized with the probe or probe set.

25. The detection method according to claim 19, wherein the detection of the hybridization is performed by analyzing a fluorescent signal with a confocal laser scanner.

26. A primer for amplifying nucleotide sequences of an enteric virus, which is selected from the group consisting of nucleotide sequences of SEQ ID NO. 19, SEQ ID NO. 23, SEQ ID NO. 24, and SEQ ID NO. 29 to SEQ ID NO. 37.

27. A detection method for an enteric virus which comprises the steps of:

(i) amplifying DNA obtained from clinical samples with primers for amplifying the enteric virus;

(ii) hybridizing the amplified DNA with a probe or probe set selected from the group consisting of nucleotide sequences of SEQ ID NO. 1 to SEQ ID NO. 15; and,

(iii) detecting the hybridization with the probe.

FIG.1A

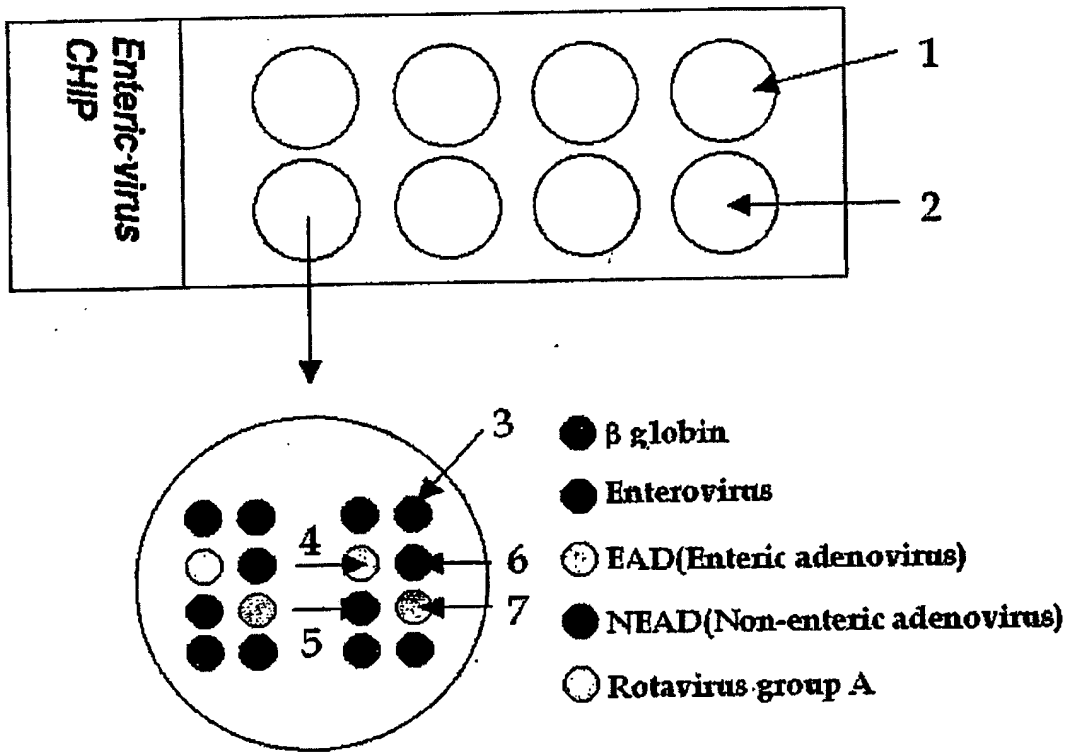

Enteric-virus CHIP
1
2
3
4
5
6
7
β globin
Enterovirus
EAD(Enteric adenovirus)
NEAD(Non-enteric adenovirus)
Rotavirus group A

FIG.1B

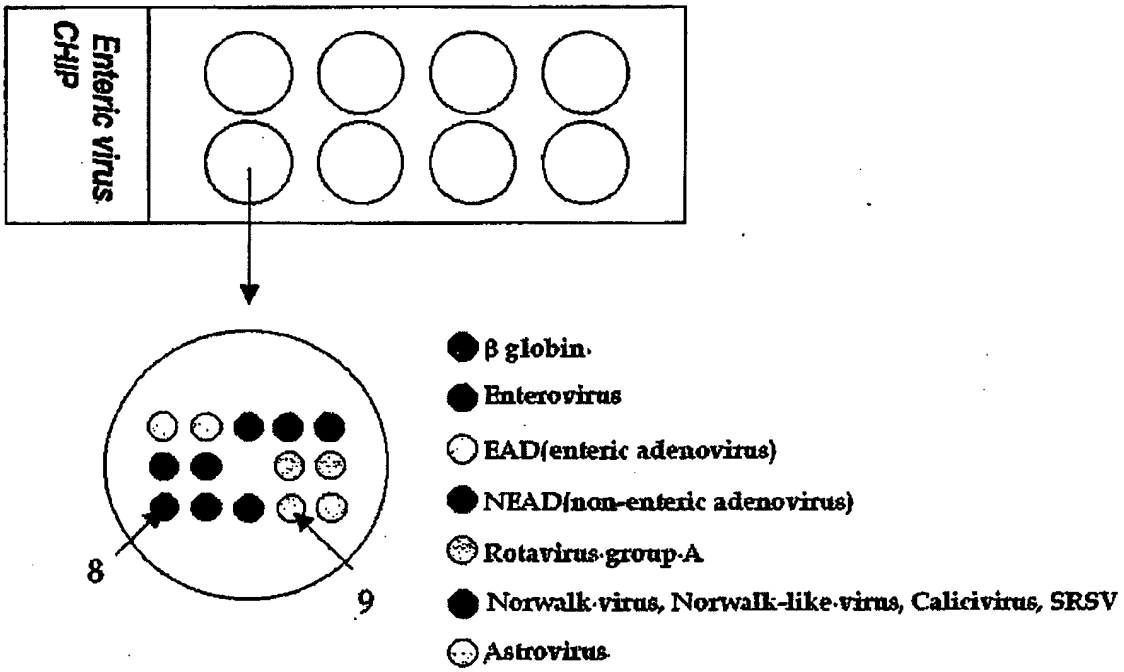
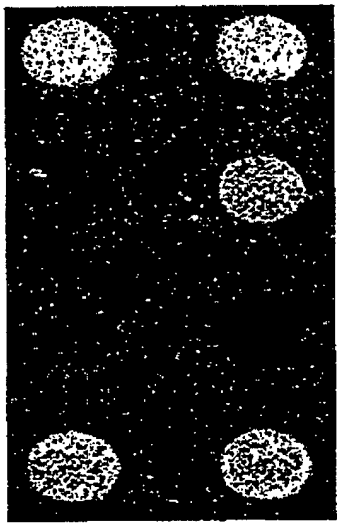

Enteric virus CHIP
8
9
β globin
Enterovirus
EAD(enteric adenovirus)
NEAD(non-enteric adenovirus)
Rotavirus group A
Norwalk virus, Norwalk-like virus, Calicivirus, SRSV
Astrovirus

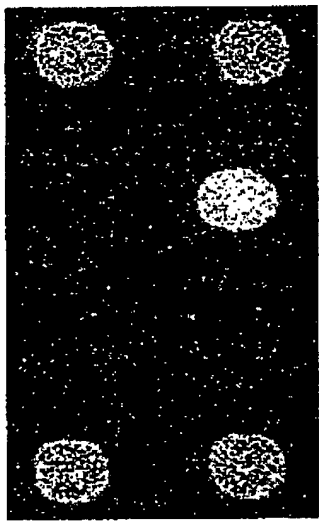
FIG.2

Concentrated water sample



A1-1-1

(source water)

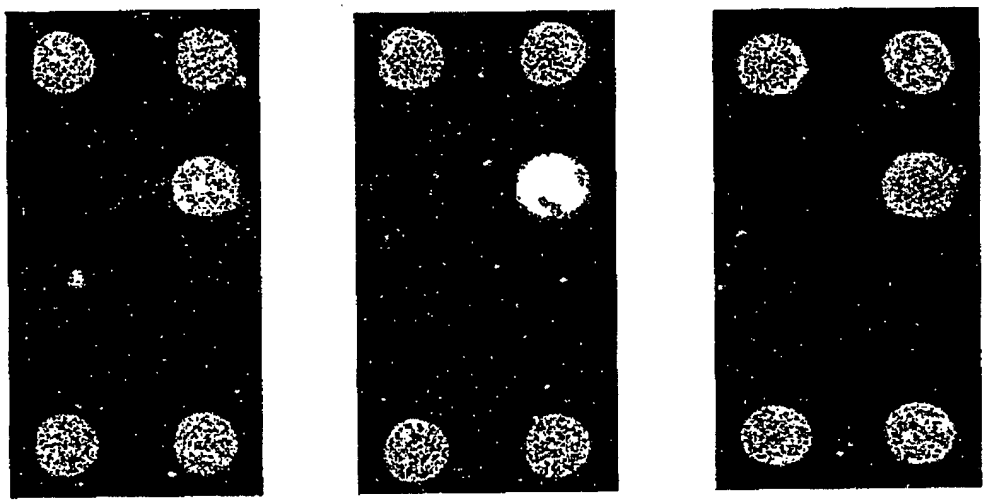


B1-2-1

(source water)

FIG.3A

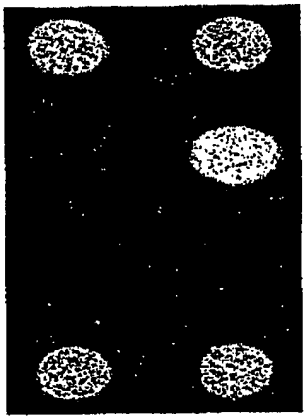
Polioviruses in Enterovirus



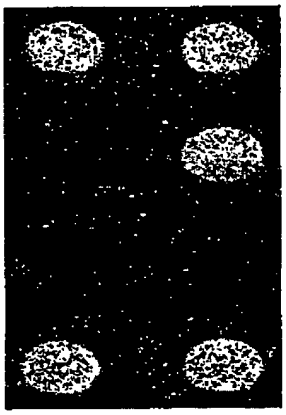
poliovirus type 1 poliovirus type 2 poliovirus type 3

FIG.3B

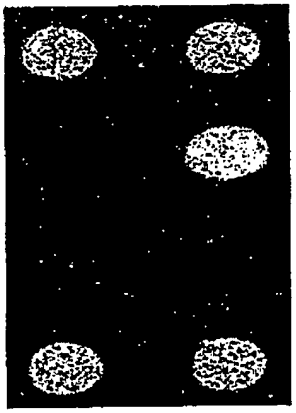
Coxsackieviruses



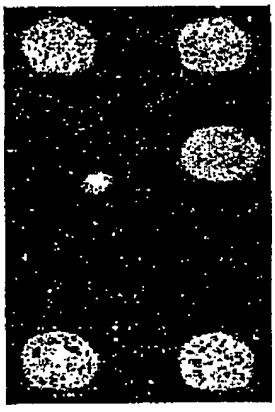
coxsackievirus B2



coxsackievirus B3



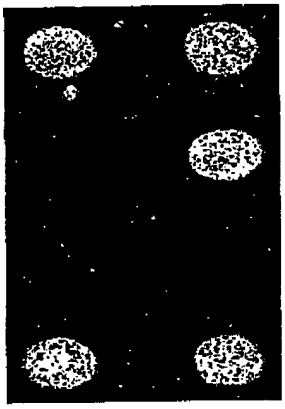
coxsackievirus B4



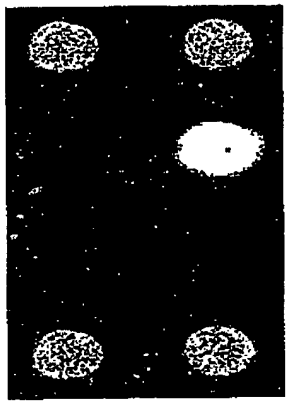
coxsackievirus B5

FIG.3C

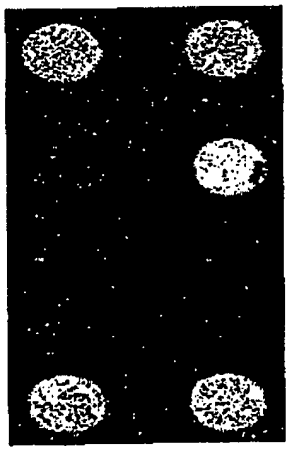
Echoviruses



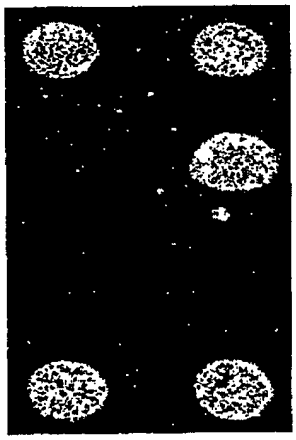
echovirus7



echovirus 11



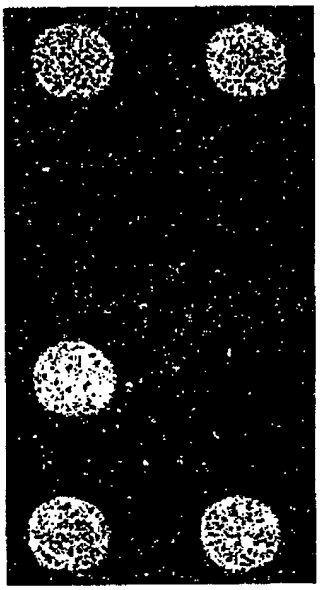
echovirus 24



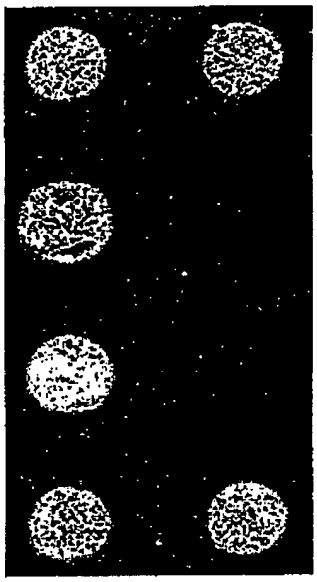
echovirus 30

FIG.4

Adenovirus



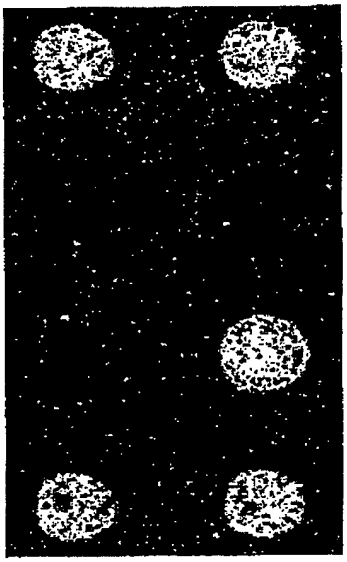
adenovirus type 5



adenovirus type 41

FIG.5

Rotavirus

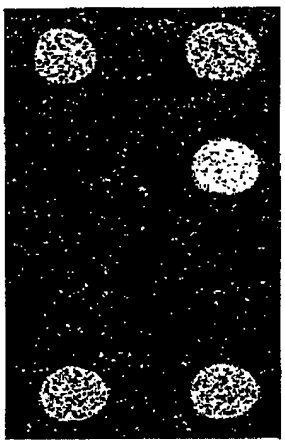


rotavirus group A

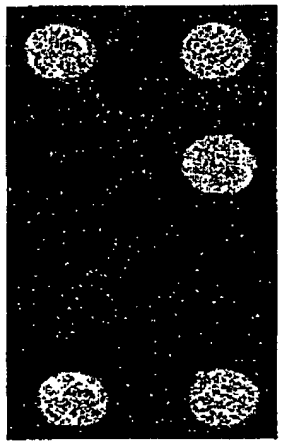
FIG.6A

<clinical specimen>

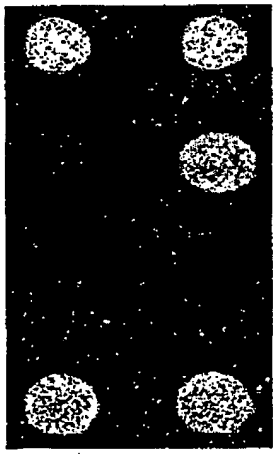
enterovirus positive stool



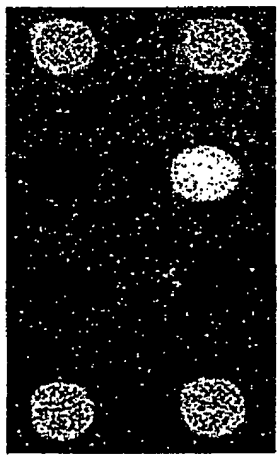
stool A



stool B



stool C

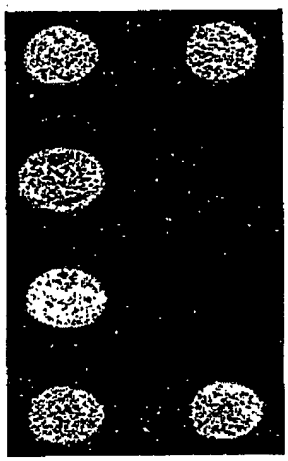


stool D

FIG.6B

adenovirus positive stool

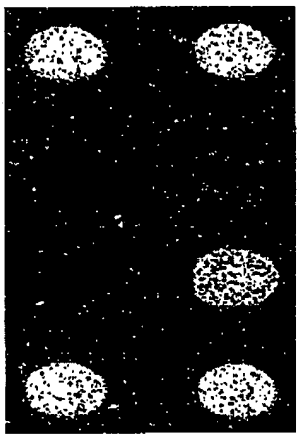
stool A(type41)



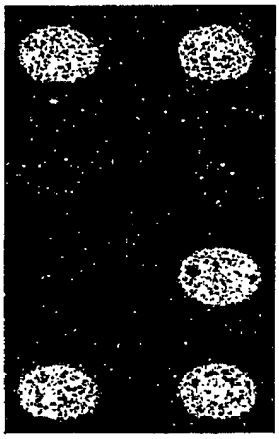
stool B(type 31)

FIG.6C

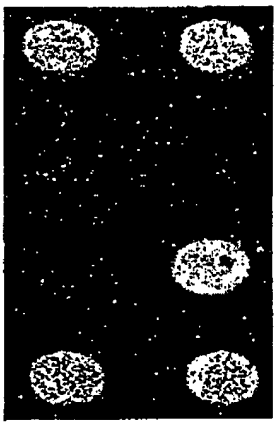
rotavirus positive stool



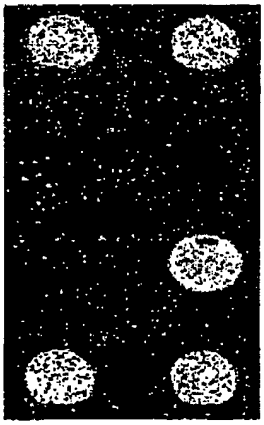
stool A



stool B



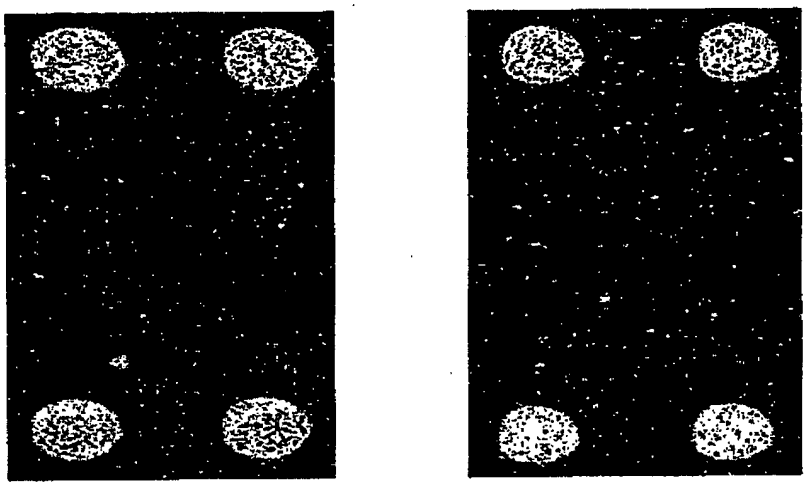
stool C



stool D

FIG.6D

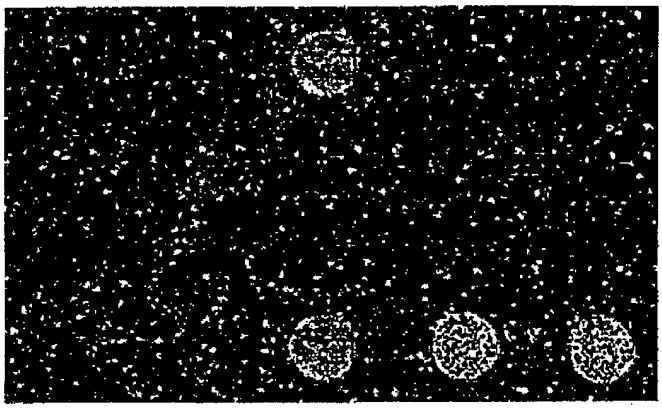
negative stool



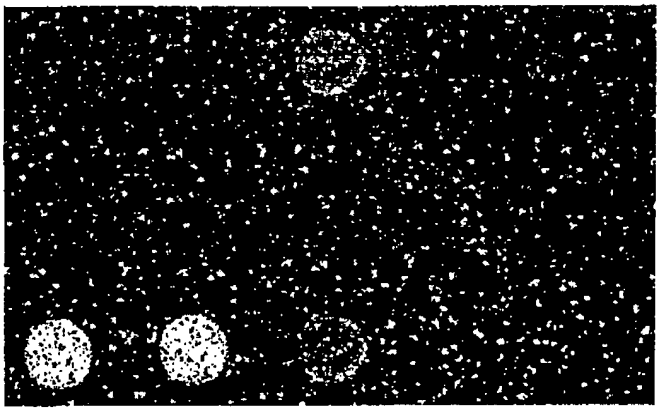
stool A stool B

FIG.7

(A) Astrovirus



(B) Caliciviruses, Norwalk viruses, Norwalk-like viruses, SRSV



<110> BIOMEDLAB CORPORATION
JEONG, Yong Seok

<120> PROBE FOR DETECTION OF ENTERIC VIRUS, DETECTION KIT AND METHOD FOR ENTERIC VIRUS WITH THE SAME

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<150> KR 10-2001-0048956
<151> 2001-08-09

<160> 37

<170> KopatentIn 1.71

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<211> 29
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<213> Artificial Sequence

<220>
<223> pan-enterovirus probe(ENTERO 1)

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<210> 2
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<212> DNA
<213> Artificial Sequence

<220>
<223> pan-enterovirus probe(ENTERO 2)

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cggaaccgac tactttgggt gtccg 25

<210> 3
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> pan-enterovirus probe(ENTERO 3)

<400> 3
ggctgcgttg gcggcctacc tatggct 27

<210> 4
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> adenovirus probe(Adeno 1)

<400> 4
gcacgcsacc cacgatgtaa ccacagacag cgtgc 35

<210> 5
<211> 35
<212> DNA
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<220>
<223> adenovirus probe(Adeno 2)

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<400> 8
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<400> 9
atggactaca taatttatag attttt 26

<210> 10
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<213> Artificial Sequence

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ctaacggtta gctcctttta atgtatggta 30

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<212> DNA
<213> Artificial Sequence

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<400> 11
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<212> DNA
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<223> Caliciviridae probe(Calici)

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<210> 13
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<223> Caliciviridae probe(Calici 1)

<400> 13
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<213> Artificial Sequence

<220>
<223> Caliciviridae probe(Calici 2)

<400> 14
aattttgtvc argcmcc 17

<210> 15
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Caliciviridae probe(Calici 3)

<400> 15
gghgarttya cnrtdtcccc 20

<210> 16
<211> 21
<212> DNA
<213> Artificial Sequence

<220>

<223> pan-enterovirus primer(EV1, 164-184, 5'-UTR)

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<210> 17
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<400> 17
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<210> 18
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<400> 18
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<210> 19
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<223> pan-enterovirus primer(EV4, 436-473, 5'-UTR)

<400> 19

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<210> 20

<211> 28

<212> DNA

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<223> Rotavirus group A primer(RV1, 1-289, casidglycoprotein VP7 segment 9)

<400> 20

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<210> 21

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> Rotavirus group A primer(RV2, 376-392, casidglycoprotein VP7 segment 9)

<400> 21

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<211> 21
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<400> 22
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<210> 23
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<400> 23
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<210> 24
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<223> Rotavirus group A primer(RV5, 292-314, casidglycoprotein VP7 segment 9)

<400> 24
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<210> 25
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> adenovirus primer(AD1, 18858-18883, ORF, hexon gene)

<400> 25
gccgcagtgg tcttacatgc acatc 25

<210> 26
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> adenovirus primer(AD2, 19136-19158, ORF, hexon gene)

<400> 26
cagcacgccg cggatgtcaa agt 23

<210> 27
<211> 24

<212> DNA
<213> Artificial Sequence

<220>
<223> adenovirus primer(AD3, 18937-18960, ORF, hexon gene)

<400> 27
gccaccgaga cgtacttcag cctg 24

<210> 28
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> adenovirus primer(AD4, 19051-19079, ORF, hexon gene)

<400> 28
ttgtacgagt acgcggtatc ctcgcggtc 29

<210> 29
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<212> DNA
<213> Artificial Sequence

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<223> human astrovirus primer (AS1, 186-204, capsid)

<400> 29
cttcccaart ttgttagg 18

<210> 30
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<212> DNA
<213> Artificial Sequence

<220>
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<400> 30
cagtaatcac aacaactcrt 20

<210> 31
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
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<210> 32
<211> 22
<212> DNA
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<220>
<223> norwalk virus, norwalk-like virus, calicivirus, SRSV primer(NV2, 295-316, capsid)

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<210> 33
<211> 21
<212> DNA
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<220>
<223> norwalk virus. norwalk-like virus, calicivirus, SRSV primer(NV3, 295-316, capsid)

<400> 33
tgccmaccca gccattatac a 21

<210> 34
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> norwalk virus. norwalk-like virus, calicivirus, SRSV primer(NV4, 342-361, capsid)

<400> 34
ccrgcngtra avgcktincc 20

<210> 35
<211> 22

<212> DNA
<213> Artificial Sequence

<220>
<223> norwalk virus, norwalk-like virus, calicivirus, SRSV primer(NV5, -4-18, capsid)

<400> 35
gtaaatgatg atggcgtcta ag 22

<210> 36
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> norwalk virus, norwalk-like virus, calicivirus, SRSV primer(NV6, 115-176, capsid)

<400> 36
ttattsatwa tccagdgrtc aa 22

<210> 37
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<212> DNA
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<220>
<223> norwalk virus, norwalk-like virus, calicivirus, SRSV primer(NV7, 184-203, capsid)

<400> 37

ggggahakng tkaaktcdcc 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR02/01530

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, PubMed, Delphion, Blast, "enterovirus", "detection", "probe", "primer"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X; Y	US 5,728,519 A (USA Department of Health and Human Sercices) 17 Mar. 1998. See the whole document.	1; 2-25, 27
Y	Toogood, C.I. et al., J. Gen Virol., 70, 3203-14, 1989. See the whole document. & NCBI Accession # X51782.	1-25, 27
Y	US 5,959,093 A (The Ohio State Univ. Research Foundation) 28 Sep. 1999. See the whole document.	1-25, 27
Y	US 4,853,333 A (American Home Products Co.) 01 Aug. 1989. See the whole document.	1-25, 27
Y	Gerna, G. et al., J. Gen Virol., 75, 1781-84, 1994. See the whole document. & NCBI Accession # L20881, L20883.	1-25, 27
Y	Mendez-Toss, M. et al., J. Gen Virol., 81, 2891-97, 2000. See the whole document. & NCBI Accession # AF260508.	1-25, 27
Y	US 5,625,049 A (Monroe, S.S. et al.) 29 Apr. 1997. See the whole document.	1-25, 27
Y	WO 01/34848 A2 (USA Department of Health and Human Sercices) 17 May 2001. See the whole document.	1-25, 27

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
24 DECEMBER 2002 (24.12.2002)	26 DECEMBER 2002 (26.12.2002)
Name and mailing address of the ISA/KR Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer LEE, Cheo Young Telephone No. 82-42-481-5594

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR02/01530

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99/06836 A1 (Tonen Co.) 11 Feb. 1999. See the whole document.	1-25, 27
Y	WO 00/58524 A2 (USA Department of Health and Human Sercices) 05 Oct. 2000. See the whole document.	1-25, 27
Y	Kawamata, O., Niigata Igakkai Zasshi, 111(10), 633-46, 1997. See abstract.	26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR02/01530

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5,728,519 A	17 Mar. 1998	none	
US 5,959,093 A	28 Sep. 1999	none	
US 4,853,333 A	01 Aug. 1989	none	
US 5,625,049 A	29 Apr. 1997	AU 6832194 A1 WO 94/26902 A1	12 Dec. 1994 24 Nov. 1994
WO 01/34848 A2	17 May 2001	AU 200112193 A5 EP 1230399 A2	06 Jun. 2001 14 Aug. 2002
WO 99/06836 A1	11 Feb. 1999	EP 967484 A1 JP 11108932 A2 JP 2001215228 A2 JP 2001226400 A2	29 Dec. 1999 23 Apr. 1999 10 Aug. 2001 21 Aug. 2001
WO 00/58524 A2	05 Oct. 2000	AU 200039167 A5 EP 1218548 A2	16 Oct. 2000 03 Jul. 2002